PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Byoung S. Kwon	Examiner: Unknown
Serial No.:	Division of 07/012,269	Group Art Unit: Unknown
Filed:	Herewith	Docket: 740.009US2
Title:	ANTIBODY FOR 4-1BB (as amended)	

PRELIMINARY AMENDMENT

Box Patent Application
Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to taking up the above-identified divisional application, please amend the application as follows:

In the Title

Please amend the title to read as follows: "ANTIBODY FOR 4-1BB".

In the Specification

Please enter the enclosed Sequence Listing into the specification.

Please substitute the paragraphs in the appendix entitled "Clean Version of Specification Paragraphs" for the paragraphs in the specification as filed. Specific amendments to individual paragraphs are detailed in the following marked up paragraphs.

Please substitute page 1, paragraph 1 for the paragraph in the appendix entitled "Clean Version of Page 1, Paragraph 1." Specific amendments to page 1, paragraph 1 are detailed in the following marked-up paragraph:

> This application is a division of copending application Serial No. 07/012,269, filed February 1, 1993, which is a continuation-in-part of copending application Serial No. 07/922,996, filed on July 30, 1992, which is a continuation-in-part of copending application Serial No. [07/267,572] 07/267,577, filed on November 7, 1988, abandoned.

Please substitute page 5, the paragraph beginning on line 22 for the paragraph in the appendix entitled "Clean Version of Page 5, the Paragraph beginning on line 22." Specific amendments to page 5, the paragraph beginning on line 22 are detailed in the following marked-up paragraph:

> A monoclonal antibody against 4-1BB was developed which specifically recognizes an epitope on the extracellular domain of receptor protein 4-1BB. The monoclonal antibody is produced from a hybridoma identified as 53A2 and deposited at the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC No.: HB-11248. The monoclonal antibody can be used to enhance T-cell proliferation by treating T-cells that have expressed receptor protein 4-1BB with antiCD3 monoclonal antibody.

Please substitute page 5, the paragraph beginning on line 29 and continuing on page 6 for the paragraph in the appendix entitled "Clean Version of Page 5, the Paragraph beginning on line 29." Specific amendments to page 5, the paragraph beginning on line 29 are detailed in the following marked-up paragraph:

> Some tumors are potentially immunogenic but do not stimulate an effective [anti-immne]anti-immune response in vivo. Tumors may be capable of delivering antigen-specific signals to T cells, but may not deliver the co-stimulatory signals necessary for full activation of T cells. Expression of the co-stimulatory ligand on B7 of melanoma cells was found to induce the rejection of a murine melanoma in vivo. ("Tumor Rejection After Direct Co-Stimulation of $CD8^+$ T Cells by B7-Transfected Melanoma Cells", Sarah E. Townsend and James P. Allison, Science Vol. 259, 1-5-93.) The monoclonal antibody of the present invention may be capable of the same effect as it is now [know]known to enduce T cell proliferation and activation.

Please substitute page 7, paragraph 2 for the paragraph in the appendix entitled "Clean Version of Page 7, Paragraph 2." Specific amendments to page 7, paragraph 2 are detailed in the following marked-up paragraph:

> Figures 2a and 2b show the nucleotide sequence (SEQ ID NO:1) and the deduced amino acid sequence (SEQ ID NO:2) of mouse receptor 4-1BB.

Please substitute page 8, paragraph 2 for the paragraph in the appendix entitled "Clean Version of Page 8, Paragraph 2." Specific amendments to page 8, paragraph 2 are detailed in the following marked-up paragraph:

Figure 17 shows a comparison of the 4-1BBP amino acid sequence (SEQ ID NO:3) with the amino acid sequence in sina (SEQ ID NO:4) [Dorsophila] Drosophila and DG17 (SEQ ID NO:5) of Dictyostelium.

Please substitute page 32, first full paragraph for the paragraph in the appendix entitled "Clean Version of Page 32, First Full Paragraph." Specific amendments to page 32, first full paragraph are detailed in the following marked-up paragraph:

> The amino-terminal sequence of the purified 4-1BBPs was determined. The sequence was Val-Gln-Asn-Ser-X-Asp (SEQ ID NO:6). The amino acid sequence at positions 1, 2, 3, 4 and 6 was identical to that of the mature 4-1BBP predicted from the cDNA sequence. Amino acid at position 5 which is supposed to be Cys was not determined. These results indicate that the deduced amino acid sequence and assignment of signal sequence are correct. When the potential transmembrane domain was removed from the complete 4-1BB molecule, the protein was secreted. These results suggested that 4-1BBP was likely to be associated with the cellular membrane as predicted by the primary structure.

Please substitute the third paragraph on page 37 continuing on page 38 for the paragraph in the appendix entitled "Clean Version of the Third paragraph on Page 37 Continuing on Page 38." Specific amendments to the third paragraph on page 37 continuing on page 38 are detailed in the following marked-up paragraph:

> Antibody Preparation. An oligopeptide representing amino acids 105-115 of the deduced 4-1BBP sequence was synthesized (Applied Biosystems). The sequence was NH_2-CRPGQELTKSGY-COOH (SEQ ID NO:7). A tyrosine residue at the C-terminus of the peptide was added for possible radioactive labeling with [^{125}I]. The peptide was conjugated to keyhole limpet hemocyanin (KLH) with a [heteroblifunctional] heterobifunctional cross linker, m-maleimidobenzoyl-n-hydroxysuccinimide ester **(88, 107)**.

Please substitute page 39, second full paragraph for the paragraph in the appendix entitled "Clean Version of Page 39, Second Full Paragraph." Specific amendments to page 39, second full paragraph are detailed in the following marked-up paragraph:

This region forms the pattern of C-X_2-C-X_9-H-X_3C-X-C (SEQ ID NO:8); and the cysteines and histidine are conserved in a similar space in 4-1BB, sina, and DG17 proteins. Ten of 24 amino acids between the 4-1BB and sina proteins are identical. Between 4-1BB and DG17 proteins, 11 of 24 amino acids are identical, and 3 of 24 are conservative substitutions. The conserved pattern suggests that these amino acids are functionally important.

Please substitute page 44, first full paragraph for the paragraph in the appendix entitled "Clean Version of Page 44, First Full Paragraph." Specific amendments to page 34, first full paragraph are detailed in the following marked-up paragraph:

4-1BB contains other interesting features in its cytoplasmic domain. Those include 1) two runs of acidic amino acids; 2) a potential $p56^{lck}$ binding site; 3) five consecutive glycines at the carboxyl terminus; and 4) four potential phosphorylation sites - 1 tyrosine, 2 threonine, and 1 serine. It is especially interesting that 4-1BB contains a potential $p56^{lck}$ binding site, -C-R-C-P- (SEQ ID NO:9). The consensus sequence of $p56^{lck}$ binding site is -C-X-C-P- (SEQ ID NO:10) in the CD4 and CD8 molecules **(93)**.

Please substitute the third paragraph on page 45 continuing on page 46 for the paragraph in the appendix entitled "Clean Version of the Third paragraph on Page 45 Continuing on page 46." Specific amendments to the third paragraph on page 45 continuing on page 46 are detailed in the following marked-up paragraph:

To construct a plasmid that expresses extracellular portion of 4-1BB, the putative extracellular domain of 4-1BB cDNA **(89)** was amplified by polymerase chain reaction (PCR) **(99)**. An XhoI site was created at the 5' end of the forward primer and a stop codon, (TAA), and an EcoRI site were created in the reverse primer. The PCR product was digested with XhoI and EcoRI and the -0.6 kb fragment was purified. The XhoI-EcoRI fragment (4-1BBS) was inserted into the PEV-55 vectors **(53)**, generating PEV-55-4-1BBS. The sequence of the forward primer (SEQ ID NO:11) was 5' -ACCTCGAGGTCCTGTGCATGT-GACA-3' and that of the reverse primer (SEQ ID NO:12) was 5' -ATGAATTCTTACTGCAGG-AGTGCCC-3'.

Please substitute page 53, the paragraph beginning on line 25 for the paragraph in the appendix entitled "Clean Version of Page 53, the Paragraph beginning on line 25." Specific amendments to page 53, the paragraph beginning on line 25 are detailed in the following marked-up paragraph:

To determine whether biochemical signals delivered through 4-1BB may contribute to T cell activation, the anti-4-1BB mAb, 53A2, was [potentiaily]potentially used to mimic ligand [billdillg]binding to cell surface 4-1BB. Purified resting splenic T cells were stimulated with 10 μg/ml immobilized anti-CD3 in the absence or presence of 53A2.

Please substitute page 55, the paragraph beginning on line 1 for the paragraph in the appendix entitled "Clean Version of Page 55, the Paragraph beginning on line 1." Specific amendments to page 53, the paragraph beginning on line 1 are detailed in the following marked-up paragraph:

In other experiments, the actual degree of enhancement ranged from an approximately [2-10fold]2-10 fold increase in [^{3}H]thymidine incorporation in [culturses]cultures stimulated with anti-CD3 alone (Table 4). Differences in actual enhancement of [^{3}H]][^{3}H] thymidine incorporation, could be due to variability in the [nunber]number or metabolic status of accessory cells in the cultures. For example, the highest-fold increase in T cells stimulated with anti-CD3 in the presence of 53A2 generally occurred when the proliferative effects of anti-CD3 were [miniml]minimal (Table 4). It will be necessary to obtain T cell populations of higher purity to address this issue. These data, however, conclusively show that 4-1BB-mediated signals can contribute to T cell [prolferation]proliferation.

Please substitute page 55, the paragraph beginning on line 15 for the paragraph in the appendix entitled "Clean Version of Page 55, the Paragraph beginning on line 15." Specific amendments to page 53, the paragraph beginning on line 15 are detailed in the following marked-up paragraph:

The major species of 4-1BB on the cell surface appears to be a 55-kDa dimer. 4-1BB also appears to exist as a 30-kDa monomer and possibly as a 110-kDa tetramer. Since these 4-1BB species were immunoprecipitated from a homogenous population of cells (T cell clone Fl), all forms potentially co-exist on each cell. A comparison of peptide digests from the 4-1BB monomer and dimer will be needed to determine whether 4-1BB exists as a homodimer on the cell surface. A variety of cell surface receptors such as the insulin receptor (**59**), the B cell surface immunoglobulin receptor (**60**), the T cell Ag [receptro]receptor (**61**), the CD28 costimulatory receptor (**62**), and the CD27 T cell antigen (**63**) are composed of disulfide-bonded subunits. Receptor dimerization may be required for ligand binding and subsequent biochemical signaling.

Please substitute page 55, the paragraph beginning on line 26 and continuing on page 56 for the paragraph in the appendix entitled "Clean Version of Page 55, the Paragraph beginning on line 26." Specific amendments to page 55, the paragraph beginning on line 26 are detailed in the following marked-up paragraph:

> 4-1BB is not expressed on resting T cells but is inducible by activators which deliver a complete growth stimulus to the T cell. The combination of PMA and ionomycin is capable of [mimicklng]mimicking those signals required for T cell proliferation. Although PMA or ionomycin alone induced 4-1BB mRNA, the combination of PMA and ionomycin resulted in optimal 4-1BB expression. Furthermore, the expression of 4-1BB was not transient. When purified splenic T cells were stimulated with immobilized anti-CD3, 4-1BB mRNA was expressed and this expression was maintained for up to 96 hrs poststimulation. Cell cycle analysis will be required to confirm that 4-1BB is expressed throughout cell cycle progression.

Please substitute page 56, the paragraph beginning on line 3 for the paragraph in the appendix entitled "Clean Version of Page 56, the Paragraph beginning on line 3." Specific amendments to page 56, the paragraph beginning on line 3 are detailed in the following marked-up paragraph:

> 4-1BB is structurally related to members of the nerve growth factor receptor super-family. Although these receptors possess structurally similar ligand-binding properties (cysteine-rich regions), the cytoplasmic domains of these proteins are nonconserved which could allow for diversity in transmembrane signaling. Some members of this family are involved in the T or B cell activation process. There are in vitro functional data on the OX-40, CD40 and CD27 antigens. Antibodies against the OX-40 angment the T cell response in a mixed lymphocyte reaction (**47**) and antibodies against CD40 enhance B-cell proliferation in the presence of a coactivator, such as PMA or CD20 antibodies, and synergize with IL-4 in vitro to induce B-cell differentiation and to generate long-term normal B cell lines (**64**). One [inonoclonal]monoclonal antibody, anti-1A4, which recognizes an epitope on the CD27 molecule inhibited calcium mobilization, IL-2 secretion, helper T cell function, and T cell proliferation. On the other hand, CLB-CD27/1, another anti-CD27 mAb enhanced proliferation of [hunan]human T cells stimulated with PHA or anti-CD3 mAb (**63**). These results indicate that the CD27 molecule plays an important role in T cell activation. Except for TNFRs, NCFR and CD40, the ligands or cell surface molecules to which the members of the superfamily bind are not yet identified. Identification and characterization of the ligands to which the receptors bind will be helpful in better defining the physiologic role of 4-1BB.

Please substitute page 57, the paragraph beginning on line 5 for the paragraph in the appendix entitled "Clean Version of Page 57, the Paragraph beginning on line 5." Specific amendments to page 57, the paragraph beginning on line 5 are detailed in the following marked-up paragraph:

> The biochemical signals delivered through 4-1BB are not completely known. One possibility considered was the observation that 4-1BB contains a putative $p56^{lck}$ tyrosine kinase binding [doimain]domain in its cytoplasmic tail. It was later determined that $p56^{lck}$ tyrosinase kinase binds to 4-1BB. It will also be worthwhile to determine if 4-1BB-mediated signaling can [regullate]regulate genes such as IL=2 and IL-2 receptor, whose expression is required for T cell activation and subsequent proliferation.

Please substitute page 58, the paragraph beginning on line 14 for the paragraph in the appendix entitled "Clean Version of Page 58, the Paragraph beginning on line 14." Specific amendments to page 58, the paragraph beginning on line 14 are detailed in the following marked-up paragraph:

> 4-1BB is a 30 kD inducible T-cell antigen, and is expressed predominantly as a 55 K dimer on both $CD4^+$ and $CD8^+$ T lymphocytes. The cytoplasmic tail of 4-1BB contains the sequence, Cys-Arg-Cys-Pro, which is [simiJar]similar to the sequence Cys-X-Cys-Pro, that mediates the binding of the CD4 and CD8 molecules to $p56^{lck}$ a protein tyrosine kinase[2,3]. An anti-4-1BB monoclonal antibody (53A2 mAb) was used to determine whether [4- 1BB]4-1BB may associate with $p56^{lck}$. The 53A2 mAb specifically recognized 4-1BB on a $CD8^+$ T-cell line, CTLL-2, and coimmunoprecipitated a 56 K protein along with 4-1BB. Peptide mapping indicated that the 56 K phosphoprotein was identical to $p56^{lck}$. The coimmunoprecipitation of $p56^{lck}$ with 4-1BB also occurred in nonlymphoid cells such as insect (Sf-21) and HeLa cells when the two recombinant proteins were coexpressed. Analysis of mutant $p56^{lck}$ recombinant proteins showed that two cysteine residues, critical for $p56^{lck}$-CD4 (or CD8) complex formation, are also required for the $P56^{lck}$-4-1BB interaction. These studies establish that 4-1BB physically associates with $p56^{lck}$.

Please substitute page 58, paragraph 1 for the paragraph in the appendix entitled "Clean Version of Page 58, Paragraph 1." Specific amendments to page 58, paragraph 1 are detailed in the following marked-up paragraph:

> This region forms the pattern of $C\text{-}X_2\text{-}C\text{-}X_9\text{-}C\text{-}X_3\text{-}H\text{-}X_3\text{-}C\text{-}X\text{-}C$ (SEQ ID NO:8); and the cysteines and histidine are conserved in a similar space in 4-1BB, *sina*, and DG17 proteins. Ten of 24 amino acids between the 4-1BB and *sina* proteins are identical, and 3 of

24, are conservative substitutes. The conserved pattern suggests that these amino acids are functionally important. The *sina* protein is localized in the nucleus, suggesting that it has a regulatory function in cells. The fact that the amino acid sequence of 4-1BB contains features like a zinc finger motif, a nuclear protein, and a receptor domain suggests that 4-1BB may play diverse roles during cellular proliferation and differentiation.

Please substitute page 58, paragraph 2 for the paragraph in the appendix entitled "Clean Version of Page 58, Paragraph 2." Specific amendments to page 58, paragraph 2 are detailed in the following marked-up paragraph:

4-1BB is a 30 kD inducible T-cell antigen, and is expressed predominantly as a 55 K dimer on both $CD4^+$ and $CD8^+$ T lymphocytes. The cytoplasmic tail of 4-1BB contains the sequence, Cys-Arg-Cys-Pro (SEQ ID NO:9), which is [simiJar] similar to the sequence Cys-X-Cys-Pro (SEQ ID NO:10), that mediates the binding of the CD4 and CD8 molecules to $p56^{lck}$ a protein tyrosine kinase[2,3]. An anti-4-1BB monoclonal antibody (53A2 mAb) was used to determine whether [4- 1BB] 4-1BB may associate with $p56^{lck}$. The 53A2 mAb specifically recognized 4-1BB on a $CD8^+$ T-cell line, CTLL-2, and coimmunoprecipitated a 56 K protein along with 4-1BB. Peptide mapping indicated that the 56 K phosphoprotein was identical to $p56^{lck}$. The comimmunoprecipitation of $p56^{lck}$ with 4-1BB also occurred in nonlymphoid cells such as insect (Sf-21) and HeLa cells when the two recombinant proteins were coexpressed. Analysis of mutant $p56^{lck}$ recombinant proteins showed that two cysteine residues, critical for $p56^{lck}$-CD4 (or CD8) complex formation, are also required for the [$P56^{lck}$-4-1BB] $p56^{lck}$-4-1BB interaction. These studies establish that 4-1BB physically associates with $p56^{lck}$.

Please substitute page 62, second full paragraph for the paragraph in the appendix entitled "Clean Version of Page 62, Second Full Paragraph." Specific amendments to page 62, second full paragraph are detailed in the following marked-up paragraph:

Figures 31a-c show an analysis of the association of 4-1BB and $p56^{lck}$ in a baculoviral expression system. Figure 31a and 31b show [and] an immunoblot of 4-1BB and $p56^{lck}$. Sf-21 [insectcellswere] insect cells were infected with 4-1BB-, $p56^{lck}$-expressing recombinant baculoviruses or coinfected with [4-1 BB] 4-1BB and $p56^{lck}$-expressing recombinant baculoviruses. Total lysates from Sf-21 cells infected with these recombinant baculoviruses were blotted and probed with rabbit anti-4-1BB and rabbit anti-$p56^{lck}$ (Fig. 31a and 31b, respectively). Antigens were visualized with [alkline] alkaline phosphatase-conjugated secondary [artibodies] antibodies and chromogenic substrates, NBT and BCIP. Anti-4-1BB polyclonal rabbit serum was raised against the oligopeptide, CRPGQELTKQG (SEQ ID NO:13), which corresponds to amino acids 82 to 92 of mature 4-1BB. [figure] Figure 31c shows an immune complex kinase assay of $p56^{lck}$. These Sf-21 cell lysates were also incubated with isotype-matched rat IgG_1 (Fig. 31c, lane 1), 53A2 (Fig. 31c, lane 2) or

anti-p56^{lck} (Fig. 31c, lane 3). The immune complexes were precipitated, subjected to the in vitro kinase reaction with [γ-^{32}P] and run on a 10% SDS-polyacrylamide gel as described in Fig. 30. The arrow indicates the autophosphorylated p56^{lck} proteins.

In the Claims

Please substitute the claim set in the appendix entitled "Clean Version of Pending Claims" for the previously pending claim set. Specific amendments to individual claims are detailed in the following marked up set of claims.

Please cancel claims 1-8 and 17-21 without prejudice.

Please add the following new claims:

22. (New) A polyclonal antibody against 4-1BB which specifically recognizes an epitope on the extracellular domain of receptor protein 4-1BB.

23. (New) The polyclonal antibody of claim 22 which specifically recognizes the epitope on residues 105-115 or 133-157 of 4-1BB.

24. (New) The monoclonal antibody of claim 9 which binds to the extracellular domain of SED NO:2.

25. (New) The polyclonal antibody of claim 22 which binds to the extracellular domain of SED NO:2

Remarks

In the Restriction Requirement dated June 20, 2000 in the parent application, Serial No. 07/012,269, to the above-identified divisional application, the Examiner grouped the claims as follows: Group I (claims 1-3, 22 and 28-30) included claims directed to a DNA encoding murine 4-1BB; Group II (claims 6-8 and 17-20) included claims directed to a 4-1BB protein, e.g., produced by recombinant means, or fragments thereof, a 4-1BB fusion protein, and a method of using the fusion protein to detect cell membrane ligands; Group III (claims 9-12) included claims directed to directed to a monoclonal antibody against 4-1BB that recognizes an epitope on the extracellular domain of 4-1BB, and a hybridoma which produces such an antibody; Group IV

(claims 13-16 and 21) included claims directed to a method of enhancing T cell proliferation or activation, or inducing B cell proliferation, with a monoclonal antibody against 4-1BB that recognizes an epitope on the extracellular domain of 4-1BB, or with cells expressing 4-1BB, respectively; and Group V (claims 23-27) included claims directed to a method to detect murine 4-1BB nucleic acid in a biological sample.

The present divisional application is directed to the claims in Group III (claims 9-12). However, as claims 9-12 are product claims, should those claims be found allowable, claims 13-16, method of use claims, all which ultimately depend from claim 9, should be rejoined with the allowed claims. MPEP 821.04.

Claims 1-8 and 17-21 are canceled and claims 22-25 are added. Claims 9-16 and 22-25 are pending.

New claims 22-23 are supported at page 26, lines 17-28 of the specification.

New claim 24 is supported by page 26, lines 17-28 and Figure 2 of the specification.

New claim 25 is supported by page 47, lines 16-26 and Figure 2 of the specification.

The title of the application is amended to more accurately reflect the claimed subject matter.

A Sequence Listing is submitted herewith to conform the above-referenced application to the requirements of 37 C.F.R. §§ 1.821 through 1.825. The paper copy of the Sequence Listing in this application is identical to the computer readable form copy of the Sequence Listing filed in application Serial No. 08/012,269, filed February 1, 1993. In accordance with 37 C.F.R. § 1.821(e), please use the computer readable form of the Sequence Listing filed on July 13, 2001 in application Serial No. 08/012,269 as the computer readable form for the instant application. A paper copy of the Sequence Listing is included with this Preliminary Amendment for incorporation into the specification.

The specification is amended to update the status of related applications, correct typographical errors, and insert reference to sequence identifier numbers.

The Examiner is respectfully requested to consider the remarks and amendments herein prior to taking up the above-identified application.

Respectfully submitted,

BYOUNG S. KWON,

By his Representatives,

SCHWEGMAN, LUNDBERG, WOESSNER & KLUTH, P.A.
P.O. Box 2938
Minneapolis, MN 55402
(612) 373-6959

Date February 4, 2002 By

Janet E. Embretson
Reg. No. 39,665

"Express Mail" mailing label number: EV041075003US
Date of Deposit: February 4, 2002
This paper or fee is being deposited on the date indicated above with the United States Postal Service pursuant to 37 CFR 1.10, and is addressed to The Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

Docket No. 00740.009US2
WD # 422900.wpd

Client Reference No. 9148 LIN

Clean Version of the Title

ANTIBODY FOR 4-1BB
Applicant: Byoung S. Kwon
Serial No.:

ANTIBODY FOR 4-1BB

Docket No. 00740.009US2 Client Reference No. 9148 LIN
WD # 422915.wpd

Clean Version of Pending Claims

ANTIBODY FOR 4-1BB
Applicant: Byoung S. Kwon
Serial No.:

---

9. A monoclonal antibody against 4-1BB which specifically recognizes an epitope on the extracellular domain of receptor protein 4-1BB.

10. The monoclonal antibody of claim 9 wherein said monoclonal antibody is produced from a hybridoma identified as 53A2 and deposited at the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC No.: HB-11248.

11. A hybridoma capable of producing a monoclonal antibody against 4-1BB which specifically recognizes an epitope on the extracellular domain of receptor protein 4-1BB.

12. The hybridoma of claim 11 wherein said hybridoma is identified as 53A2 and deposited at the American type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC No.: HB-11248.

13. The method of using the monoclonal antibody of claim 9 to enhance T-cell proliferation comprising the step of treating T-cells that have expressed receptor protein 4-1BB with said monoclonal antibody.

14. The method of claim 13 further comprising the step of conducting said treatment in the presence of protein tyrosinase kinase.

15. The method of using the monoclonal antibody of claim 9 to enhance T-cell activation comprising the strep of treating T-cells that have expressed receptor protein 4-1BB with said monoclonal antibody.

16. The method of claim 15 further comprising the step of conducting said treatment in the presence of protein tyrosinase kinase.

22. (New) A polyclonal antibody against 4-1BB which specifically recognizes an epitope on the extracellular domain of receptor protein 4-1BB.

23. (New) The polyclonal antibody of claim 22 which specifically recognizes the epitope on residues 105-115 or 133-157 of 4-1BB.

24. (New) The monoclonal antibody of claim 9 which binds to the extracellular domain of SED NO:2.

25. (New) The polyclonal antibody of claim 22 which binds to the extracellular domain of SED NO:2

Docket No. 00740.009US2
WD # 422901.wpd

Client Reference No. 9148 LIN

Clean Version of Specification Paragraphs

ANTIBODY FOR 4-1BB
Applicant: Byoung S. Kwon
Serial No.:

Page 1, paragraph 1:

This application is a division of copending application Serial No. 07/012,269, filed February 1, 1993, which is a continuation-in-part of copending application Serial No. 07/922,996, filed on July 30, 1992, which is a continuation-in-part of copending application Serial No. 07/267,577, filed on November 7, 1988, abandoned.

Page 5, the paragraph beginning on line 22:

A monoclonal antibody against 4-1BB was developed which specifically recognizes an epitope on the extracellular domain of receptor protein 4-1BB. The monoclonal antibody is produced from a hybridoma identified as 53A2 and deposited at the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC No.: HB-11248. The monoclonal antibody can be used to enhance T-cell proliferation by treating T-cells that have expressed receptor protein 4-1BB with antiCD3 monoclonal antibody.

Page 5, the paragraph beginning on line 29 and continuing on page 6:

Some tumors are potentially immunogenic but do not stimulate an effective anti-immune response in vivo. Tumors may be capable of delivering antigen-specific signals to T cells, but may not deliver the co-stimulatory signals necessary for full activation of T cells. Expression of the co-stimulatory ligand on B7 of melanoma cells was found to induce the rejection of a murine melanoma in vivo. ("Tumor Rejection After Direct Co-Stimulation of $CD8^+$ T Cells by B7-Transfected Melanoma Cells", Sarah E. Townsend and James P. Allison, Science Vol. 259, 1-5-93.) The monoclonal antibody of the present invention may be capable of the same effect as it is now known to enduce T cell proliferation and activation.

Page 7, paragraph 2:

Figures 2a and 2b show the nucleotide sequence (SEQ ID NO:1) and the deduced amino acid sequence (SEQ ID NO:2) of mouse receptor 4-1BB.

Page 8, paragraph 2:

Figure 17 shows a comparison of the 4-1BBP amino acid sequence (SEQ ID NO:3) with the amino acid sequence in sina (SEQ ID NO:4) Drosophila and DG17 (SEQ ID NO:5) of Dictyostelium.

Page 32, first full paragraph:

The amino-terminal sequence of the purified 4-1BBPs was determined. The sequence was Val-Gln-Asn-Ser-X-Asp (SEQ ID NO:6). The amino acid sequence at positions 1, 2, 3, 4 and 6 was identical to that of the mature 4-1BBP predicted from the cDNA sequence. Amino acid at position 5 which is supposed to be Cys was not determined. These results indicate that the deduced amino acid sequence and assignment of signal sequence are correct. When the potential transmembrane domain was removed from the complete 4-1BB molecule, the protein was secreted. These results suggested that 4-1BBP was likely to be associated with the cellular membrane as predicted by the primary structure.

The third paragraph on page 37 continuing on page 38:

Antibody Preparation. An oligopeptide representing amino acids 105-115 of the deduced 4-1BBP sequence was synthesized (Applied Biosystems). The sequence was NH_2-CRPGQELTKSGY-COOH (SEQ ID NO:7). A tyrosine residue at the C-terminus of the peptide was added for possible radioactive labeling with [^{125}I]. The peptide was conjugated to keyhole limpet hemocyanin (KLH) with a heterobifunctional cross linker, m-maleimidobenzoyl-n-hydroxysuccinimide ester **(88, 107)**.

Page 39, second full paragraph:

This region forms the pattern of $C\text{-}X_2\text{-}C\text{-}X_9\text{-}H\text{-}X_3C\text{-}X\text{-}C$ (SEQ ID NO:8); and the cysteines and histidine are conserved in a similar space in 4-1BB, sina, and DG17 proteins. Ten of 24 amino acids between the 4-1BB and sina proteins are identical. Between 4-1BBand DG17 proteins, 11 of 24 amino acids are identical, and 3 of 24 are conservative substitutions. The conserved pattern suggests that these amino acids are functionally important.

Page 44, first full paragraph :

4-1BB contains other interesting features in its cytoplasmic domain. Those include 1) two runs of acidic amino acids; 2) a potential $p56^{lck}$ binding site; 3) five consecutive glycines at the carboxyl terminus; and 4) four potential phosphorylation sites - 1 tyrosine, 2 threonine, and 1 serine. It is especially interesting that 4-1BB contains a potential $p56^{lck}$ binding site, -C-R-C-P- (SEQ ID NO:9). The consensus sequence of $p56^{lck}$ binding site is -C-X-C-P- (SEQ ID NO:10) in the CD4 and CD8 molecules **(93)**.

The third paragraph on page 45 continuing on page 46:

To construct a plasmid that expresses extracellular portion of 4-1BB, the putative extracellular domain of 4-1BB cDNA **(89)** was amplified by polymerase chain reaction (PCR) **(99)**. An XhoI site was created at the 5' end of the forward primer and a stop codon, (TAA), and an EcoRI site were created in the reverse primer. The PCR product was digested with XhoI and EcoRI and the -0.6 kb fragment was purified. The XhoI-EcoRI fragment (4-1BBS) was inserted into the PEV-55 vectors **(53)**, generating PEV-55-4-1BBS. The sequence of the forward primer (SEQ ID NO:11) was 5' -ACCTCGAGGTCCTGTGCATGT-GACA-3' and that of the reverse primer (SEQ ID NO:12) was 5' -ATGAATTCTTACTGCAGG-AGTGCCC-3'.

Page 53, the paragraph beginning on line 25:

To determine whether biochemical signals delivered through 4-1BB may contribute to T cell activation, the anti-4-1BB mAb, 53A2, was potentially used to mimic ligand binding to cell surface 4-1BB. Purified resting splenic T cells were stimulated with 10 μg/ml immobilized anti-CD3 in the absence or presence of 53A2.

Page 55, the paragraph beginning on line 1:

In other experiments, the actual degree of enhancement ranged from an approximately 2-10 fold increase in [^{3}H]thymidine incorporation in cultures stimulated with anti-CD3 alone (Table 4). Differences in actual enhancement of [^{3}H] thymidine incorporation, could be due to variability in the number or metabolic status of accessory cells in the cultures. For example, the highest-fold increase in T cells stimulated with anti-CD3 in the presence of 53A2 generally occurred when the proliferative effects of anti-CD3 were minimal (Table 4). It will be necessary to obtain T cell populations of higher purity to address this issue. These data, however, conclusively show that 4-1BB-mediated signals can contribute to T cell proliferation.

Page 55, the paragraph beginning on line 15:

The major species of 4-1BB on the cell surface appears to be a 55-kDa dimer. 4-1BB also appears to exist as a 30-kDa monomer and possibly as a 110-kDa tetramer. Since these 4-1BB species were immunoprecipitated from a homogenous population of cells (T cell clone Fl), all forms potentially co-exist on each cell. A comparison of peptide digests from the 4-1BB monomer and dimer will be needed to determine whether 4-1BB exists as a homodimer on the cell surface. A variety of cell surface receptors such as the insulin receptor (**59**), the B cell surface immunoglobulin receptor (**60**), the T cell Ag receptor (**61**), the CD28 costimulatory receptor (**62**), and the CD27 T cell antigen (**63**) are composed of disulfide-bonded subunits. Receptor dimerization may be required for ligand binding and subsequent biochemical signaling.

Page 55, the paragraph beginning on line 26 and continuing on page 56:

4-1BB is not expressed on resting T cells but is inducible by activators which deliver a complete growth stimulus to the T cell. The combination of PMA and ionomycin is capable of mimicking those signals required for T cell proliferation. Although PMA or ionomycin alone induced 4-1BB mRNA, the combination of PMA and ionomycin resulted in optimal 4-1BB expression. Furthermore, the expression of 4-1BB was not transient. When purified splenic T cells were stimulated with immobilized anti-CD3, 4-1BB mRNA was expressed and this expression was maintained for up to 96 hrs poststimulation. Cell cycle analysis will be required to confirm that 4-1BB is expressed throughout cell cycle progression.

Page 56, the paragraph beginning on line 3:

4-1BB is structurally related to members of the nerve growth factor receptor super-family. Although these receptors possess structurally similar ligand-binding properties (cysteine-rich regions), the cytoplasmic domains of these proteins are nonconserved which could allow for diversity in transmembrane signaling. Some members of this family are involved in the T or B cell activation process. There are in vitro functional data on the OX-40, CD40 and CD27 antigens. Antibodies against the OX-40 angment the T cell response in a mixed lymphocyte reaction (**47**) and antibodies against CD40 enhance B-cell proliferation in the presence of a coactivator, such as PMA or CD20 antibodies, and synergize with IL-4 in vitro to induce B-cell differentiation and to generate long-term normal B cell lines (**64**). One monoclonal antibody, anti-1A4, which recognizes an epitope on the CD27 molecule inhibited calcium mobilization, IL-2 secretion, helper T cell function, and T cell proliferation. On the other hand, CLB-CD27/1, another anti-CD27 mAb enhanced proliferation of human T cells stimulated with PHA or anti-CD3 mAb (**63**). These results indicate that the CD27

molecule plays an important role in T cell activation. Except for TNFRs, NCFR and CD40, the ligands or cell surface molecules to which the members of the superfamily bind are not yet identified. Identification and characterization of the ligands to which the receptors bind will be helpful in better defining the physiologic role of 4-1BB.

Page 57, the paragraph beginning on line 5:

The biochemical signals delivered through 4-1BB are not completely known. One possibility considered was the observation that 4-1BB contains a putative $p56^{lck}$ tyrosine kinase binding domain in its cytoplasmic tail. It was later determined that $p56^{lck}$ tyrosinase kinase binds to 4-1BB. It will also be worthwhile to determine if 4-1BB-mediated signaling can regulate genes such as IL=2 and IL-2 receptor, whose expression is required for T cell activation and subsequent proliferation.

Page 58, the paragraph beginning on line 14:

4-1BB is a 30 kD inducible T-cell antigen, and is expressed predominantly as a 55 K dimer on both $CD4^+$ and $CD8^+$ T lymphocytes. The cytoplasmic tail of 4-1BB contains the sequence, Cys-Arg-Cys-Pro, which is similar to the sequence Cys-X-Cys-Pro, that mediates the binding of the CD4 and CD8 molecules to $p56^{lck}$ a protein tyrosine kinase[2,3]. An anti-4-1BB monoclonal antibody (53A2 mAb) was used to determine whether 4-1BB may associate with $p56^{lck}$. The 53A2 mAb specifically recognized 4-1BB on a $CD8^+$ T-cell line, CTLL-2, and coimmunoprecipitated a 56 K protein along with 4-1BB. Peptide mapping indicated that the 56 K phosphoprotein was identical to $p56^{lck}$. The coimmunoprecipitation of $p56^{lck}$ with 4-1BB also occurred in nonlymphoid cells such as insect (Sf-21) and HeLa cells when the two recombinant proteins were coexpressed. Analysis of mutant $p56^{lck}$ recombinant proteins showed that two cysteine residues, critical for $p56^{lck}$-CD4 (or CD8) complex formation, are also required for the $P56^{lck}$-4-1BB interaction. These studies establish that 4-1BB physically associates with $p56^{lck}$.

Page 58, paragraph 1:

This region forms the pattern of C-X_2-C-X_9-C-X_3-H-X_3-C-X-C (SEQ ID NO:8); and the cysteines and histidine are conserved in a similar space in 4-1BB, *sina*, and DG17 proteins. Ten of 24 amino acids between the 4-1BB and *sina* proteins are identical, and 3 of 24, are conservative substitutes. The conserved pattern suggests that these amino acids are functionally important. The *sina* protein is localized in the nucleus, suggesting that it has a regulatory function in cells. The fact that the amino acid sequence of 4-1BB contains features like a zinc finger motif, a nuclear protein, and a receptor domain suggests that 4-1BB may play diverse roles during cellular proliferation and differentiation.

Page 58, paragraph 2:

4-1BB is a 30 kD inducible T-cell antigen, and is expressed predominantly as a 55 K dimer on both $CD4^+$ and $CD8^+$ T lymphocytes. The cytoplasmic tail of 4-1BB contains the sequence, Cys-Arg-Cys-Pro (SEQ ID NO:9), which is similar to the sequence Cys-X-Cys-Pro (SEQ ID NO:10), that mediates the binding of the CD4 and CD8 molecules to $p56^{lck}$ a protein tyrosine kinase[2,3]. An anti-4-1BB monoclonal antibody (53A2 mAb) was used to determine whether 4-1BB may associate with $p56^{lck}$. The 53A2 mAb specifically recognized 4-1BB on a $CD8^+$ T-cell line, CTLL-2, and coimmunoprecipitated a 56 K protein along with 4-1BB. Peptide mapping indicated that the 56 K phosphoprotein was identical to $p56^{lck}$. The comimmunoprecipitation of $p56^{lck}$ with 4-1BB also occurred in nonlymphoid cells such as insect (Sf-21) and HeLa cells when the two recombinant proteins were coexpressed. Analysis of mutant $p56^{lck}$ recombinant proteins showed that two cysteine residues, critical for $p56^{lck}$-CD4 (or CD8) complex formation, are also required for the $p56^{lck}$-4-1BB interaction. These studies establish that 4-1BB physically associates with $p56^{lck}$.

Page 62, second full paragraph:

Figures 31a-c show an analysis of the association of 4-1BB and $p56^{lck}$ in a baculoviral expression system. Figure 31a and 31b show an immunoblot of 4-1BB and $p56^{lck}$. Sf-21 insect cells were infected with 4-1BB-, $p56^{lck}$-expressing recombinant baculoviruses or coinfected with 4-1BB and $p56^{lck}$-expressing recombinant baculoviruses. Total lysates from Sf-21 cells infected with these recombinant baculoviruses were blotted and probed with rabbit anti-4-1BB and rabbit anti-$p56^{lck}$ (Fig. 31a and 31b, respectively). Antigens were visualized with alkaline phosphatase-conjugated secondary antibodies and chromogenic substrates, NBT and BCIP. Anti-4-1BB polyclonal rabbit serum was raised against the oligopeptide, CRPGQELTKQG (SEQ ID NO:13), which corresponds to amino acids 82 to 92 of mature 4-1BB. Figure 31c shows an immune complex kinase assay of $p56^{lck}$. These Sf-21 cell lysates were also incubated with isotype-matched rat IgG_1 (Fig. 31c, lane 1), 53A2 (Fig. 31c, lane 2) or anti-$p56^{lck}$ (Fig. 31c, lane 3). The immune complexes were precipitated, subjected to the in vitro kinase reaction with [γ-^{32}P] and run on a 10% SDS-polyacrylamide gel as described in Fig. 30. The arrow indicates the autophosphorylated $p56^{lck}$ proteins.